A METHOD TO DETERMINE BRITISH ANTI-LEWISITE IN PLASMA UTILIZING GAS CHROMATOGRAPHY/MASS SPECTROMETRY

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ABSTRACT

British anti-Lewisite (BAL) (2,3-dimercapto-1-propanol) is a potential therapeutic compound against the effects of cutaneous sulfur mustard, and a method for its determination in plasma has been developed. BAL and the internal standard (IS) ethane dithiol were removed from plasma samples through solid phase extraction, then reacted with 1-pentafluoropropionylimidazole (PFPI), forming stable pentafluoropropionyl derivates that are sensitive to gas chromatography/mass spectroscopy (GC/MS) analysis. Examining concentrations versus peak area ratios of BAL and IS derivatives demonstrated the method to be linear from 0.48 to 124 ng/ml in plasma with acceptable accuracy and precision. This method has proven to be both a reliable and sensitive means to determine BAL in plasma.

INTRODUCTION

British anti-Lewisite (BAL), or 2,3-dimercapto-1-propanol, was developed prior to the end of World War II for treatment against Lewisite gas exposure, and is still used clinically as a chelating agent against acute heavy metal poisoning[1]. BAL forms a stable water-soluble cyclic complex with heavy metals (arsenite, As^{+3}; in the case of lewisite poisoning), allowing excretion from the body. For the most part, disposition studies following the treatment of heavy metal intoxication focus on examining the elimination of stable BAL-heavy metal complexes. However, little is known about the concentration-time course of the free drug in plasma. Recent studies have indicated that BAL administration has the potential to reduce dermal inflammation subsequent to sulfur mustard exposure. To better understand the relationship between the efficacy of BAL and plasma concentrations, the pharmacokinetics of the compound need to be examined and characterized.

The dithiol BAL is difficult to analyze in plasma because of extensive binding to proteins due to rapid formation of disulfide bridges with thiol groups of cysteine residues[2]. We have utilized dithiothreitol (DTT) to cleave the disulfide bridges BAL forms with plasma proteins. The reduced and unbound BAL was then extracted from the plasma via solid phase extraction (SPE) cartridges and subsequently derivatized with 1-pentafluoropropionylimidazole (PFPI). Analysis was accomplished utilizing gas chromatographic/mass spectrometric (GC/MS) detection of the stable pentafluoropropionyl derivative of BAL, allowing both selective and sensitive detection of the compound from plasma.

EXPERIMENTAL

All chemicals and solvents were of HPLC grade or higher. The internal standard ethane dithiol (EDT) and BAL were purchased from Sigma Chemical Co. (St. Louis, MO) and DTT was obtained from Fluka (Switzerland). Plasma was separated from swine (*Sus scrofa*) whole blood obtained from Archer Farms Inc. (Darlington, MD). Pentafluoropropionylimidazole was obtained from Sigma Chemical Co. and Pierce Chemical Co (Rockford, IL). Oasis (HLB; Hydrophilic-Lipophilic Balance) SPE Cartridges (30 mg HLB sorbent) were purchased from Waters Corporation (Milford, MA).

Gas chromatographic separations were performed on an Agilent 6890 Series II gas chromatograph

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equipped with a DB-5MS bonded phase column (30 m x 0.25 mm i.d.; 0.25 μm film thickness, J & W Scientific, Folsom, CA). The initial oven temperature of 50° C was held for 2 min, then ramped to 150 °C at 10 °C min^{-1}, and finally increased to 280 °C at 40 °C min^{-1}. An Agilent 7673 autosampler introduced 1 μl into a 265 °C injection port with splitless injection, and an inlet purge of 50 ml min^{-1} was begun 1 minute post-injection. The inlet liner was an Agilent deactivated borosilicate single-taper with glass wool packing. The GC transfer line was held at 265 °C.

The GC was interfaced with an Agilent 5973N mass selective detector for conducting electron impact MS analysis. The MS analysis was conducted using selected ion monitoring (SIM) mode for both pentafluoropropionyl derivatives of EDT (*m/z* 193, 206, 207) and BAL (*m/z* 203, 219, 382, 383) with a dwell time of 50 ms each, resulting in a total scan time of 6.94 cycles min^{-1}. The MS conditions were as follows: ion source pressure approximately 1.5 x 10^{-5} torr, source temperature 230°C, electron energy 70 eV, electron emission current 50 μA, and the electron multiplier voltage +400 V relative to the autotune setting.

METHODS

Stock solutions of BAL (12.4 μg/ml) for use in standard curves and the internal standard (IS) EDT (0.725 μg/ml) were prepared in acetonitrile, while DTT (100 mM) was prepared in water. All were stored at 4°C. Dithiothreitol (30 μl) was added to each 255 μl plasma sample with gentle shaking for 1 min. Ethane dithiol (5 μl) was then added with an additional 30 seconds of shaking resulting in an IS concentration of 12.5 ng/ml. SPE cartridges were conditioned with 1 ml of methanol followed by 1 ml of water. The resulting 290 μl sample was then split into duplicate 145 μl aliquots that were passed through two separate SPE cartridges at a 1 ml min^{-1} flow rate. The SPE cartridges were washed with 1 ml of water and dried by applying vacuum for 20 min (15 in-Hg or 7.4 psi). The samples were eluted from the cartridge with the addition of 750 μl of methylene chloride, further dried with the addition of anhydrous sodium sulfate (approximately 150 mg), and spun at 2000 rpm for 5 min. The methylene chloride was decanted into a fresh vial where 10 μl of PFPI acylation reagent was added and shaken for 5 min. The reaction was quenched with the addition of 1 ml of water with 45 seconds of vigorous shaking. The samples were spun at 2,000 rpm for 5 min after which the organic layer was removed for GC/MS analysis.

The method was optimized for the recovery of BAL, as measured by the area under the curve (AUC) of PFPI-derivatized product subsequent to GC/MS analysis. The two variables examined to accomplish this optimization were the concentration and reaction times of DTT and PFPI. The cleavage of BAL from plasma proteins was addressed by varying both the volume (5, 15, 25, 35 μl) and concentration of DTT added to plasma samples, and by examining different reaction times (1, 5, 15, 30, 60, 120 min). For PFPI, the volume (5, 10, 15, 20 μl) of PFPI added to the extracted and dried methylene chloride, and the optimal reaction times (5, 15, 30, 60, 120 min) were examined.

Standard curves were prepared and analyzed on seven separate days as follows. Plasma (255μl) spiked with 124 ng/ml BAL was serial diluted with equal volumes of fresh plasma. Final standard curve concentrations of 0.48, 0.97, 1.94, 3.88, 7.75, 15.5, 31.0, 62.0, and 124.0 ng/ml were processed as described previously. For each mass chromatogram, the area under the curve (AUC) was determined for both BAL and IS peaks. A weighted regression line ($1/y^2$) was generated from the mean AUC ratios (BAL/IS) as a function of actual concentration, and was used to quantify samples with unknown BAL concentrations.

The accuracy and precision of this method was assessed by examining the intra- and inter-day variability of eight unknown test samples (0.727, 1.45, 2.91, 5.81, 11.63, 23.25, 46.50, and 93.0 ng/ml). For inter-day studies, plasma test samples were prepared and analyzed each day over a period of six days. Intra-day studies used the same concentrations as those for inter-day experiments, however all concentrations were prepared and analyzed the same day. Precision was measured by calculating the coefficient of variation (CV) for each group of test sample concentrations. Accuracy was expressed as percent error by examining differences between the calculated and the expected concentrations

[(calculated-expected)/expected x 100].

Stability was examined utilizing the same concentrations as those for the standard curve studies. Sample stability was assessed at three stages of sample preparation and under storage conditions of 4° C or frozen at -70° C. The first stage examined the stability of BAL in plasma. Second, the stability of BAL subsequent to SPE in dry methylene chloride was observed. Finally, the stability of the pentafluoropropionyl derivative of BAL was assessed. All samples, other than the stored derivatized BAL, were derivatized every other day for fourteen days.

To demonstrate the utility of the assay, preliminary studies examining the concentration-time course of BAL in hairless guinea pigs were conducted. Plasma concentrations of BAL were determined using the described assay following *im* administration of the compound. In preliminary experiments, a time-course profile was produced by examining blood samples collected at 0, 2, 10, 15, 30, 60, and 120 minutes post-BAL administration for doses of 50, 25, 5.0, 2.5, 0.5, 0.25 mg/kg (n=1). Subsequent studies at doses 0.05 and 0.025 mg/kg (n=3) included additional blood samples at 1, 240 and 360 minutes. Immediately following the collection of the terminal blood sample, plasma was separated and examined as described.

RESULTS

Chemical structures for BAL and EDT and the proposed pentafluoropropionyl derivatives of these compounds are presented in Figure 1. A tri-substituted derivative is illustrated in the case of BAL and di-substituted for EDT, along with the proposed fragmentation patterns for each.

Figure 1. **Proposed fragmentation pattern**

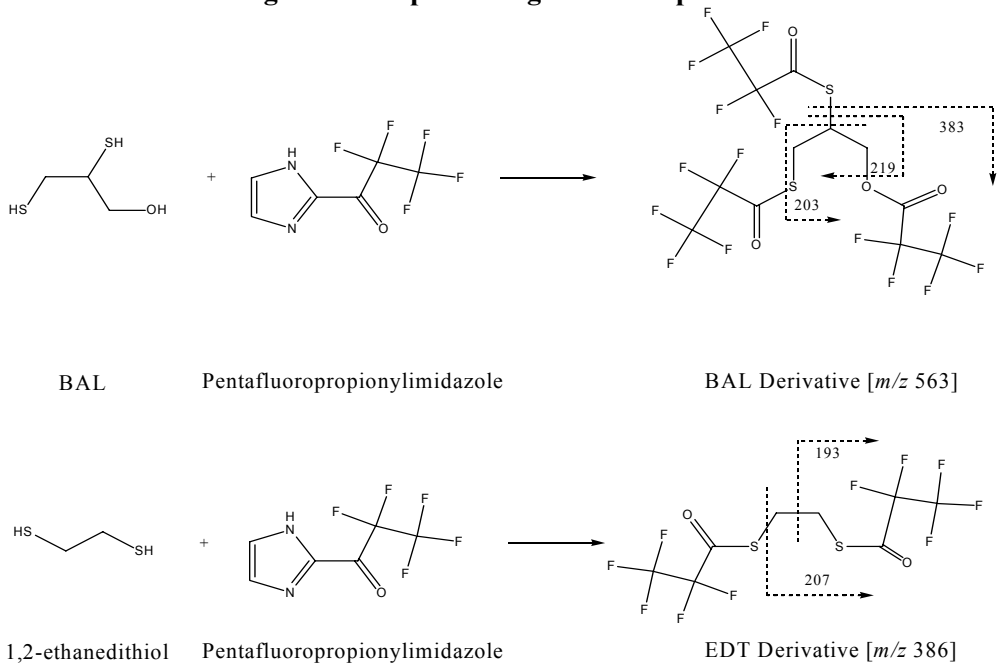

Figure 1. The chemical structures of BAL, EDT, PFPI, and the resulting pentafluoropropionyl derivative products are depicted. The proposed fragmentation pattern is also shown.

Figure 2 illustrates a representative mass-chromatogram of BAL and EDT extracted from hairless guinea pig plasma and derivatized with PFPI. The solid line represents the initial control blood sample (time 0), while the dashed line represents 120 min post-*im* administration of BAL (0.05 mg/kg) and resulting concentration of 10.1 ng/ml. The chromatogram demonstrates that BAL and EDT are adequately separated and free from confounding peaks.

Figure 2: Representative Mass Chromatogram

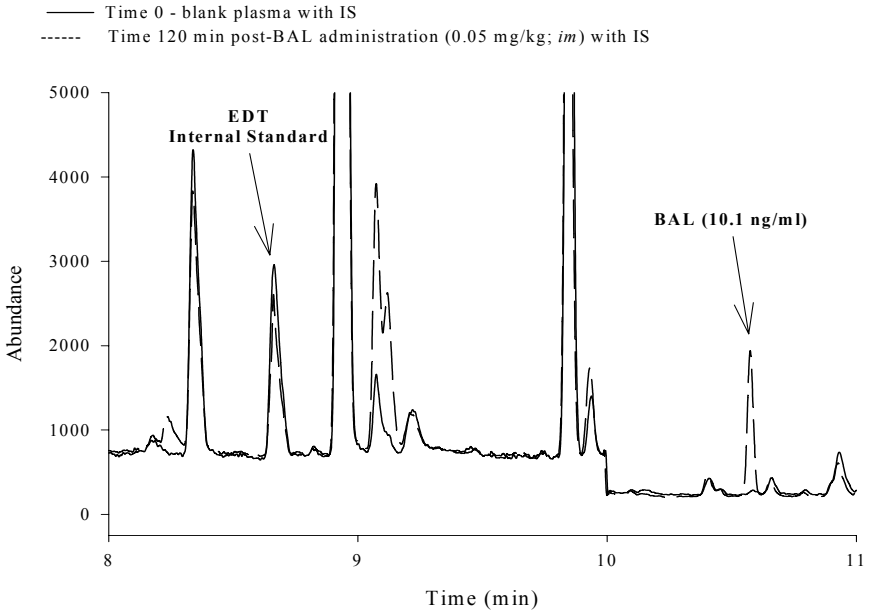

Figure 2. Shown is a representative mass-chromatogram of BAL and EDT extracted from hairless guinea pig plasma and derivatized with PFPI. The solid trace represents the initial control blood sample (time 0), while the dashed line represents 120 min post-*im* administration of BAL (0.05 mg/kg) and a resulting concentration of 10.1 ng/ml.

For this method, PFPI and DTT were optimized for both volume and reaction times according to the amount of BAL recovered from plasma, as measured by MS abundance. For PFPI, it was found that a 10 ul addition was the optimal amount added to the extracted and dried methylene chloride. The 10 µl volume of PFPI was chosen because it resulted in an 8% increase in BAL abundance over the next highest abundance found with 5 µl. The PFPI-BAL derivatization reaction was shown to occur within 5 minutes at room temperature and BAL abundance remained constant from 5-15 min. At 30 min recovered BAL decreased significantly ($p<0.05$), while the standard error of mean (SEM) at 120 min increased 300% compared to the SEM at 5 min. The 5 minute reaction time was chosen because it provided the shortest time for adequate formation of the BAL-PFPI product.

The optimal volume of DTT added to the BAL-exposed plasma was 30 µl, while the amount of time for the DTT to cleave the disulfide bridges of the BAL-protein complex seemed to occur at less than 1 minute. The 30 µl addition was chosen since it was found to be the smallest amount of DTT to provide maximum BAL recovery. Increased DTT concentrations did little to increase recovery but caused additional interfering peaks in the area of interest. The DTT reaction occurred at less than 1 minute, with abundances at 1, 5, and 15 min being statistically equivalent. Times beyond 15 min (30, 60, 120) were statistically different for BAL recovery with commensurate increases in variability ($p<0.05$). Based on these observations, one minute was chosen as the DTT reaction time for the procedure.

The mean ratio (BAL/EDT) versus concentration standard curve data shown in Figure 3 is fit to a weighted ($1/y^2$) least-squares regression analysis ($r^2 = 0.9981$) with the resulting equation of the line. The method showed linearity over a concentration range of 0.48 to 124.0 ng/ml in plasma with correlation coefficients of 0.9995 to 0.9943 for seven runs and CV's of 2.5 to 8.7% over eight concentrations tested.

Figure 3: Standard Curve

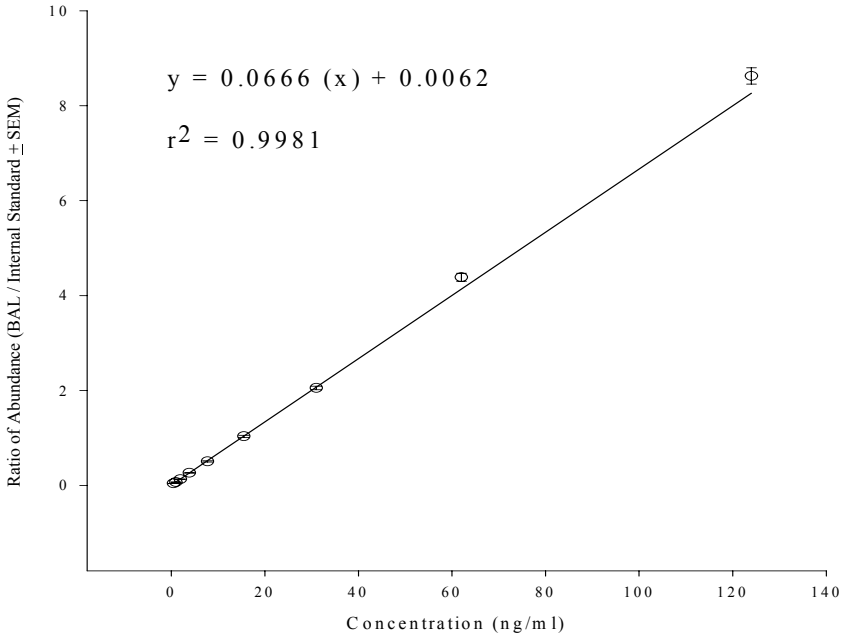

Figure 3. Standard curve with weighted ($1/y^2$) least-squares regression analysis, the resulting equation of the line, and correlation coefficient of 0.9981are shown. Data represent mean values of each standard curve (n=7) and the corresponding standard error of the mean.

Accuracy and precision data for intra- and inter-day plasma samples are presented in Table 1. For intra-day samples the accuracy, as expressed by percent error, ranged from 0.21 to -15.4%, while the precision, as expressed by %CV, was less than 9.8% over all sample concentrations (n=6). Inter-day test unknown samples results were similar in that the accuracy was shown to be 0.4 to -7.1%, while precision was 4.7 to 9.5% (n=6).

Table 1: Precision and Accuracy

Expected Concentration	Intra-day			Inter-day		
	Mean Calculated Concentration (ng/ml)	CV (%)	Error (%)	Mean Calculated Concentration (ng/ml)	CV (%)	Error (%)
0.727	0.728	7.0	0.21	0.68	7.4	-6.1
1.45	1.24	9.8	-14.6	1.26	8.9	-5.9
2.91	2.55	5.3	-12.1	2.43	8.6	-6.2
5.81	5.21	8.6	-10.3	5.28	4.7	-5.3
11.63	10.38	8.9	-10.7	10.07	9.5	-6.1
23.25	19.60	2.9	-15.4	20.52	6.2	-7.1
46.50	40.77	6.4	-12.3	41.82	8.1	-3.8
93.0	85.62	7.9	-7.9	87.89	7.2	0.4

Table 1. The precision and accuracy of the method is presented. For inter-day studies, plasma test samples were prepared and analyzed each day over a period of six days. Intra-day studies used the same concentrations as those for inter-day experiments, however all concentrations were prepared and analyzed the same day. Precision was measured by calculating the coefficient of variation (CV) for each group of test sample concentrations. Accuracy was expressed as percent error by examining differences between the calculated and the expected concentrations [(calculated-expected)/expected x 100].

The BAL stored in frozen plasma (-70º C) remained unchanged in each of the eight concentrations tested for more than 14 days, with CV's less than 10%. Solutions of derivatized BAL and EDT in methylene chloride were also stable for 14 days, with CV's of less than 10% when stored at either +4°C or -70°C. Non-derivatized solutions of BAL and EDT in methylene chloride were stable for up to one week with CV's less than 10% at both +4°C and -70°C.

The mean plasma concentration-time profile for BAL at 0.05 and 0.025 mg/kg in hairless guinea pigs following *im* administration is presented in Figure 4. This plot represents mean concentrations ± SEM at each dose. A dose response was also observed in additional preliminary studies examining 50, 25, 5.0, 2.5, 0.5, and 0.25 mg/kg administered *im* (n=1).

Figure 4: Time-Course of BAL in Hairless Guinea Pigs Post-Administration *im*

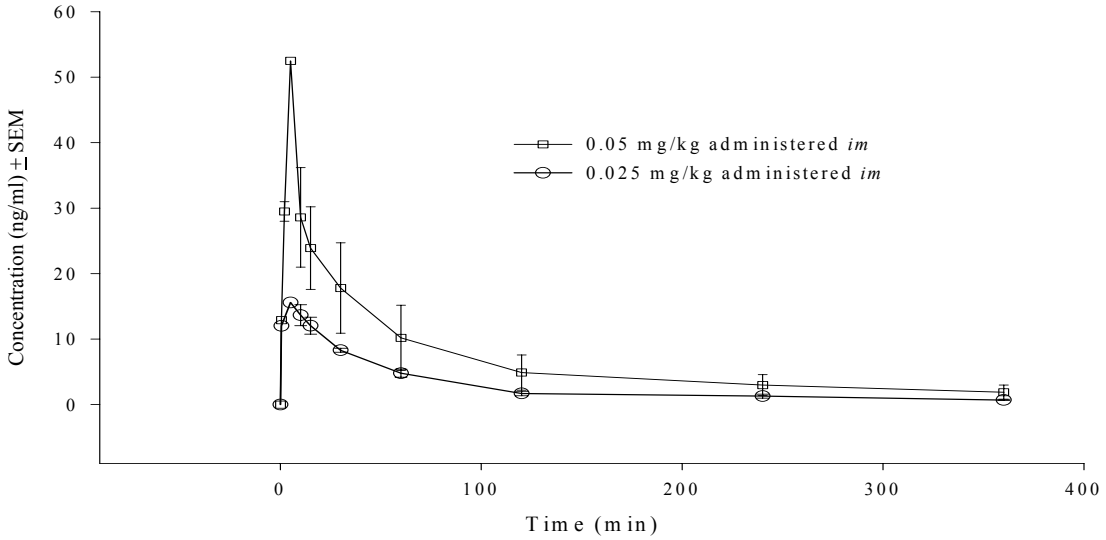

Figure 4. Plots represent the preliminary studies investigating the concentration-time profile of BAL after *im* administration in hairless guinea pigs (n=3). Hairless guinea pigs received 0.05 or 0.025 mg/kg at time 0; afterwards serial blood samples were obtained and treated as described in *Methods*.

CONCLUSIONS

A novel GC/MS method for the detection of BAL in plasma has been developed. The method has been shown to be reproducible, selective, and sensitive for the PFPI derivative of BAL. The utility of the method has been demonstrated by examining the pharmacokinetics of BAL using serial blood sampling after im administration to Hairless guinea pigs.

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